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EVALUATION AND MODIFICATION OF PLASMID DNA PURIFICATION KITS

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13. ABSTRACT *(Maximum 200 words)*

Rapid polymerase chain reaction (PCR) amplification of DNA in small volumes requires more stringent controls of DNA purity. Although DNA extracted using commercial kits meets purity requirements, they are time consuming and inefficient. Kit protocols were modified to not only remove PCR inhibitors, but also to increase efficiency. Using modified commercial plasmid purification kits, we were able to amplify plasmid DNA extracted from field samples containing humic acid, a well known PCR inhibitor. The DNA extracted was amplified using the LightCycler, a hot air thermal cycler that uses cycle times of <1min/cycle and provides real time detection of target material using dsDNA dye intercalation followed by melting curve analysis.

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PREFACE

The work described in this report was authorized under Project No. 611384/ACB1, Non-Medical CB Defense. The work was started in March 1998 completed in September 1998.

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1. INTRODUCTION

Identification of bacteria using DNA has the advantage over other methods in that it allows for absolute identification not only of indigenous organisms, but also of genetic material isolated from genetically engineered organisms (GEM). DNA technology for specific organism identification has made many advances from bench-top polymerase chain reaction (PCR) amplification using water baths, to devices that perform PCR and DNA detection on a microchip. Current laboratory testing requires the use of purified DNA, especially in microchip arrays[1,2].

Microfluidics and microchemistry require highly purified DNA. As instrumentation is miniaturized for microfluidic molecular systems (microflumes) and microelectromechanical devices (MEM), many new challenges have arisen. DARPA's Microflumes Program, supporter of rapid, miniaturized PCR and DNA detection, has identified sample cleanup followed by rapid DNA extraction as a major challenge for the future. Present research is focusing on areas of microfluidics, micromachining, microchemistry and surface chemistry; only one paper presented at a recent meeting addressed the challenge of cleanup by use of a gravity/dielectrophoresis device, which was previously reported[3]. Since any DNA sample injected into the system must be free of both particulate material and soluble inhibitors, it is imperative that this challenge be addressed simultaneously. Purified DNA is required as starting material for the suitcase-sized miniature analytical thermal cycling instrument (MATCI) which has been used to amplify human and spirochete DNA, and Hantavirus RNA[4-5]. DNA from white blood cells, however, has been successfully amplified with lysis occurring during the PCR cycles[6]. It is important to note that DNA derived from tissue is much easier to extract than that from other sources and contains few inhibitors.

The two major challenges for future DNA detection in field samples are 1) developing rapid methods of simultaneously producing pure DNA and removing PCR-inhibiting materials and, 2) developing rapid DNA detection methods. This "collection-to-detection" procedure should use few steps, not require the use of toxic or hazardous materials, be inexpensive and be adaptable to microchip technology. The purpose of this paper is to present rapid bacterial DNA detection methods using modified protocols.

2. MATERIALS AND METHODS

2.1 Plasmid DNA Purification.

Plasmid DNA was extracted from a recombinant *E. coli* by following manufacturers instructions for each kit. The starting material was 3-4 ml of bacteria culture and was purified using the following steps: 1) bacteria removal by centrifugation and resuspension, 2) alkaline lysis of cells and DNA denaturization, 3) neutralization and plasmid renaturization, 4) plasmid binding to filter or resin and plasmid elution. These kits are specifically developed for purification of plasmid DNA. RNase is added in the resuspension buffer to remove RNA; genomic DNA is removed during the renaturization step. Kits were tested and results compared (Table 1).

Table 1. Profile of DNA Purification Using Plasmid Kits

Manufacturer	Method	Purification time	*Total DNA µg/ml	DNA purity, A_{260}/A_{280}
Amersham	DNA-binding resin	28 min.	63 113	1.51 1.95
Bio-Rad	Diatomaceous earth	30 min.	73 88	1.72 1.77
Boehringer-Mannheim	Glass filter fleece	40 min.	136 119	1.89 1.63
CPG Inc.	DNA-binding resin	24 min.	89 106	1.62 1.65
MoBio	silica membrane spin filter	19 min.	124 130	1.85 1.66
Omega Biotek	proprietary matrix	38 min.	81 118	1.39 1.55
Pharmacia Biotech	Glass fiber matrix	23 min.	117 152	1.62 1.71
5-Prime, 3-Prime	DNA-binding matrix	25 min.	60 63	1.38 1.19
Qiagen	Silica-gel membrane	30 min.	46 70	1.36 1.64
Clontech	Silica membrane filter	40 min.	129 107	1.78 1.68
Promega	Silica membrane	37 min.	48 --	1.01 --
!GeneReleaser (no RNase)	Anion resin	5 min	380	2.09
GeneReleaser	Anion resin	5 min	300	1.79

*Warburg-Christian Concentration, first number is without addition of humic acid, second number, humic acid added to the culture prior to purification.

!Starting volume of bacteria culture was 5-10 µl.

2.2 Protocol Modifications.

Use of DNA-binding substrate results in only about 50% of the DNA being purified. Attempts to improve the yield while retaining requisite purity were done by separation in Sephadex G-25 M column (PD-10, Pharmacia Biotech). The column was equilibrated with 25 ml TE, sample added and eluted with 1 ml fractions of TE. Fraction 4 contained the majority of the eluted DNA and was used for PCR amplification.

Previous attempts to use GeneReleaser (GR) for use with the LightCycler thermal cycler were unsuccessful, presumably due to the presence of RNA in the sample (see Table 1). RNase A was added to the GR cleanup process to determine the efficiency of that addition.

2.3 Instrumentation.

LightCycler™ A20 (LC, Idaho Technology, Idaho Falls, ID) was used to amplify target DNA following cleanup. This instrument is a hot air thermal cycler with run times of 30 cycles from 50 min using PCR tubes to 15 min using capillary cuvettes. An additional advantage to the Lightcycler™ is its built-in fluorometer which allows for real-time monitoring of target DNA production.

2.4 PCR Protocol.

PCR Mastermix (Mg^{++} 2 mM), 50 pmol each forward and reverse primers, bovine serum albumin (BSA, 2.5 mg/ml), SYBR Green I (1:10,000 dilution) and purified plasmid DNA (10% v:v) were mixed, transferred to microcuvettes and run for 45 cycles in the Lightcycler. Table 2 summarizes the PCR temperature/time cycles.

Table 2. PCR Temperature/Time Cycle

Step	Temperature		Time
Initial denaturation	94°C		30 sec
PCR Cycles (45)			
Denaturation-	94°C		0 sec
Annealing	55°C	(30+ 0.5%GC primer)	3 sec
Extention	72°C	(0.03x product length)	26 sec
Melting curve			
denaturation	95°C		0 sec
	70°C		60 sec
	60°C		0 sec
ramp 0.2 sec/°	94°C		4 sec

2.5 DNA Detection.

Real-time detection of LC-amplified DNA was accomplished by the use of double strand (ds) DNA-specific dye, SYBR® Green I (Molecular Probes, Eugene, OR). This dye binds dsDNA and its fluorescence is enhanced by binding. As target DNA is produced during the PCR cycles, the dye binds, giving a fluorescent signal that is proportional to the amount of DNA. DNA melts at a characteristic temperature called the melting temperature (T_m), and is defined as the temperature at which half of the DNA helical structure is lost. The melting of DNA is dependent upon GC content, length of target, and sequence; hence, nonspecific primer dimer melts at a temperature that is significantly different from that of the target[7].

3. RESULTS

The concentration and purity of DNA from the commercial kits was sufficient for use in the LC. Figure 1 shows a typical melting curve from one of the PCR amplifications.

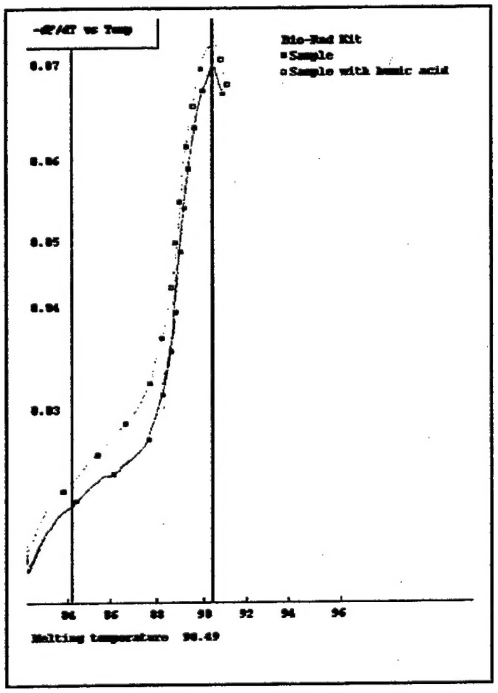
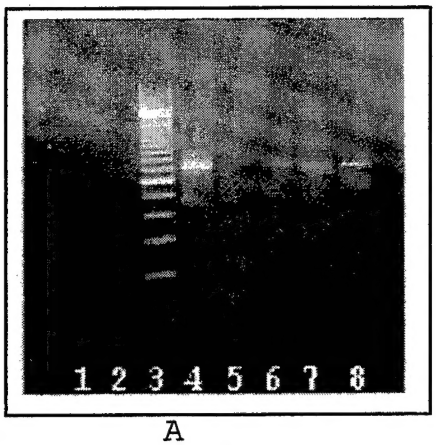


Figure 1. Melting curve analysis of Bio-Rad purification kit, with (□) and without (■) addition of humic acid.

The addition of RNase to the GR cleanup also removed the inhibiting RNA as can be seen by the purity level in Table 1 (1.79), and melting curve analysis from the LC was verified by gel electrophoresis (Figure 2).



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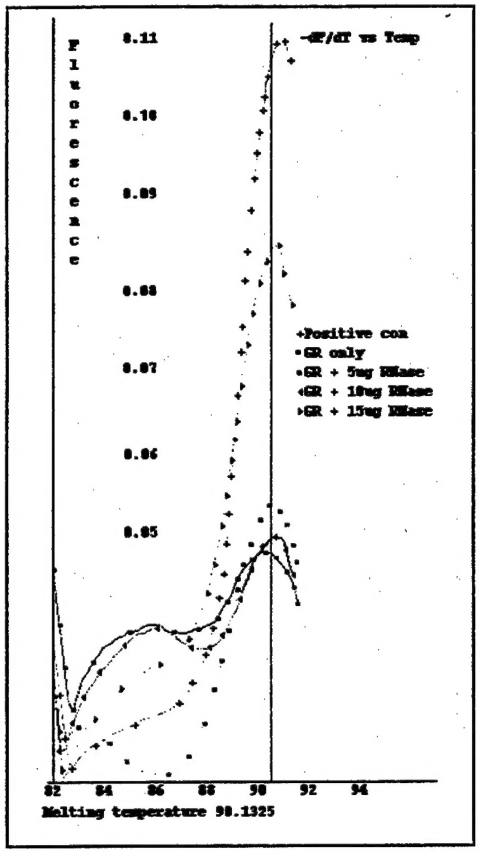

B

Figure 2. Addition of RNase to GR for purification. (A) Lane 1- GR w/o RNase, 2- negative control, 3- bp markers, 4- positive control, 5- GR, 6- GR + 5 µg RNase, 7- GR+ 10 µg RNase, 8- GR + 15 µg RNase. (B) LC melting curve analysis of amplified DNA.

Use of a PD-10 column for buffer exchange also yielded DNA that amplified in the LC. Purity analysis indicates that while the concentration was >10X more than commercial kit purification, the level of protein was high (Table 3). This did not interfere with LC amplification.

Table 3. Comparison of DNA Purification Methods

Method	A_{260}/A_{280}	Nucleic acid conc.	Protein conc.
PD-10	1.45	107 µg/ml	871 µg/ml
Spin filter	2.01	8 µg/ml	2 µg/ml

4. DISCUSSION

The ideal preparation for sample cleanup would be one in which the inhibitors present in the sample are removed and no inhibitors added by the process. It would also produce DNA of sufficient concentration and purity to eliminate further cleanup steps. Although this is possible with a simple matrix, current sample cleanup from complex matrices requires multiple steps. In

addition to the above, soil samples contain indigenous bacterial DNA which may interfere with small numbers of the organism of interest. The processing time for PCR-based detection in complex matrix often takes more than an hour, and remains too long to allow for rapid cleanup. In addition, DNA purity becomes more critical with the use of small PCR volume and microchip technology.

The advantages to using GR are that it is rapid, inexpensive, requires one-step and uses no hazardous material. Inhibitors found in soil and food samples remain a major challenge, where future testing will focus. Additional steps such as magnetic bead separation of bacteria and a modified GR cleanup will most likely be required.

This hot air thermal cycler has the advantage of real time fluorescent monitoring using dsDNA dyes and uses capillary cuvettes with a reaction volume of only 10 µl. DNA purified by a commercial plasmid extraction kit was successfully amplified using this instrument.

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